Caspase Activation Correlates With the Degree of Inflammatory Liver Injury in Chronic Hepatitis C Virus Infection

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Hepatitis C virus (HCV) infection is a major cause of liver disease characterized by inflammation, cell damage, and fibrotic reactions of hepatocytes. Apoptosis has been implicated in the pathogenesis, although it is unclear whether proteases of the caspase family as the central executioners of apoptosis are involved and how caspase activation contributes to liver injury. In the present study, we measured the activation of effector caspases in liver biopsy specimens of patients with chronic HCV infection. The activation of caspase-3, caspase-7, and cleavage of poly(ADP-ribose)polymerase (PARP), a specific caspase substrate, were measured by immunohistochemistry and Western blot analysis by using antibodies that selectively detect the active truncated, but not the inactive precursor forms of the caspases and PARP. We found that caspase activation was considerably elevated in liver lobules of HCV patients in comparison to normal controls. Interestingly, the immunoreactive cells did yet not reveal an overt apoptotic morphology. The extent of caspase activation correlated significantly with the disease grade, *i.e.*, necroinflammatory activity. In contrast, no correlation was observed with other surrogate markers such as serum transaminases and viral load. In biopsy specimens with low activity (grade 0) 7.7% of the hepatocytes revealed caspase-3 activation, whereas 20.9% of the cells stained positively in grade 3. Thus, our results suggest that caspase activation is involved in HCV-associated liver injury. Moreover, measurement of caspase activity may represent a reliable marker for the early detection of liver damage, which may open up new diagnostic and therapeutic strategies in HCV infection. (HEPATOLOGY 2001;34:758-767.)

Hepatitis C virus (HCV) infection is one of the major causes of liver disease with an increased risk of cirrhosis and hepatocellular carcinoma. The infection has a high propensity to chronicity, and the majority of HCV carriers have histologic evidence for liver inflammation, cell damage, and fibrotic reactions of hepatocytes. The mechanisms responsible for HCV-mediated liver cell damage are poorly understood, and both immune-mediated reactions and direct cytopathic effects of HCV may be involved in its pathogenesis. It has been suggested that apoptosis plays an important role in HCV-associated liver injury,[1-4] although it is unclear which cellular and molecular mechanisms participate in the process.

One of the best-defined apoptotic pathways is mediated by the death receptor CD95, a member of the tumor necrosis factor superfamily that is constitutively expressed on hepatocytes.[5] Experiments in mice have shown that agonistic CD95 antibodies cause massive liver cell lysis, resulting in increased serum levels of transaminases and death from fulminant hepatic failure.[6] In patients with chronic HCV infection, expression of CD95 is increased and associated with disease activity and the severity of liver inflammation.[7,8] When HCV-specific T cells migrate towards hepatocytes and recognize viral antigens through the T-cell receptor, they become activated and inducibly express the ligand CD95L that can transduce the apoptotic death signal to CD95-bearing hepatocytes.[2] In addition to CD95L and other cytokines, both structural and nonstructural HCV proteins have been shown to modulate the sensitivity of hepatocytes for cell death.[9-13]

Cells undergoing apoptosis show a sequence of morphologic features including membrane blebbing, cellular shrinkage, and condensation of chromatin. Recent studies showed that the key morphologic alterations of apoptosis are essentially mediated by a family of conserved intracellular proteases, called caspases.[14-17] In mammalian cells, at least 14 different caspase members exist that are cysteine proteases that cleave several cellular substrates after aspartate residues. Caspases are synthesized as inactive proenzymes and proteolytically processed to constitute an active complex composed of 2 heterodimeric subunits of about 10 and 20 kd. There is ample evidence that caspases form an intracellular proteolytic cascade that serves to amplify the apoptotic signal. Based on their structure and order in cell death pathways, caspases can be divided into initiator and effector caspases. Initiator caspases, such as caspase-8 and -9, exert regulatory roles. Upon binding to signal-transducing molecules they activate downstream effector caspases, such as caspase-3, -6, or -7, which finally cleave different cellular substrates, thereby inducing the apoptotic cell death. Among different substrates are enzymes involved in genome function such as the DNA repair enzyme poly(ADP-ribose)polymerase (PARP), regulators of the cell cycle such as retinoblastoma protein and

Abbreviations: HCV, hepatitis C virus; PARP, poly(ADP-ribose)polymerase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; dUTP, deoxyuridine triphosphate.

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MDM-2, and structural proteins of the nucleus and cytoskeleton including lamins, gesolins, and cytokeratins.[18] Furthermore, DNA cleavage is triggered on caspase-mediated degradation of the inhibitory subunit of an endonuclease, designated CAD for caspase-activated DNase.[19]

Despite the rapid elucidation of apoptotic signaling, it is almost unknown whether and to what extent caspases are activated in human pathologies. Recently, we showed that caspases play a role in various liver diseases.[20] In the present study, we investigated the activation of caspases in liver biopsy specimens from patients with chronic HCV infection using novel antibodies specific for the activated form of caspase-3 and -7 as well as for the cleaved caspase substrate PARP. We show that HCV-mediated liver damage is characterized by increased activation of effector caspases. The extent of caspase activation correlated with the grade of disease, but not with surrogate markers, such as serum transaminases or viral load. Thus, our data suggest that activation of caspases and apoptosis are involved in the HCV-mediated liver damage.

PATIENTS AND METHODS

Cells and Reagents. The human hepatoma cell line HepG2 was maintained in Dulbecco's modified Eagle medium, 10% fetal calf serum, 100 units of penicillin/mL, and 0.1 mg streptomycin/mL (all from Gibco BRL, Eggenstein, Germany). The agonistic anti-CD95 antibody (IgG3) was received from BioCheck (Münster, Germany). The activation-specific polyclonal antisera against cleaved caspase-7 and cleaved caspase-3 were purchased from NEB (Beverly, MA) and RDS (Wiesbaden, Germany), respectively. The antisera were produced by immunization of rabbits with KLH-coupled synthetic peptides around the cleavage sites of caspase-3 and -7, and therefore detected the active large subunits of the respective caspase, but not the inactive precursor forms. The monoclonal cleavage site-directed anti-PARP antibody was kindly provided by Dr. G. Fertig (Roche Molecular Research, Penzberg, Germany) and Dr. M. Brockhaus (Roche Diagnostics, Basel, Switzerland). It was generated by immunization of mice with a peptide surrounding the PARP caspase cleavage site $DEVD_{214}$. The antibody was specific for a caspase-generated neoepitope of the p85 fragment of PARP, but did not recognize the full-length protein. To detect the full-length forms of caspases and PARP, mouse-anti-caspase-3 (Transduction Laboratory, Heidelberg, Germany), mouse anti–caspase-7 monoclonal antibody (Pharmingen, Heidelberg, Germany) and a rabbit anti-PARP antiserum (Roche Molecular Research) were used. Biotinylated horse anti-rabbit IgG, anti-mouse IgG, and peroxidase-conjugated avidin-biotin complex were obtained from Vector Laboratories (Burlingame, CA). Cy3- and phycoerythrine-conjugated donkey anti-rabbit and anti-mouse IgG antibodies were from Jackson Laboratories and purchased from Dianova (Hamburg, Germany). Cycloheximide, bovine serum albumin (BSA), 3-amino-9-ethyl-carbazole (AEC), and hematoxylin were purchased from Sigma (Deisenhofen, Germany).

Patients. We investigated 20 patients (9 women, 11 men, 22-66 years, mean age 44.4 ± 12.2) with chronic HCV infection. The study was performed with informed consent from each patient according to the guidelines of the ethics committee of the University of Münster. The diagnosis was based on histologic examination by 2 pathologists as well as on the presence of anti-HCV antibodies and HCV RNA in serum for at least 6 months. The patients had no other causes of liver disease and did not receive any HCV-specific therapy at the time of investigation. The presence of anti-HCV antibodies was determined by a commercially available enzyme-linked immunosorbent assay kit (Abbott Diagnostics, Wiesbaden, Germany). The amount of HCV RNA in serum samples was quantified by the Amplicor HCV Monitor test following the manufacturer's instructions (Roche Diagnostics). Genotyping of HCV according to the classification of Simmonds[21] was performed by a reverse hybridization assay (Inno LiPA HCV II; Innogenetics, Ghent, Belgium). In 2 cases the virus load was not determined, and in 6 cases the genotype was unknown. The histologic diagnosis was established using hematoxylin-eosin and Masson trichrome stains of formalin-fixed paraffin-embedded liver obtained at the same time as the specimens used for the immunohistochemical analyses. The liver biopsy specimens in the experimental group were assessed by 2 pathologists in a double-blinded fashion as to the grade of disease activity and stage of fibrosis according to Batts and Ludwig.[22] The necroinflammatory grade was based on morphologic features of portal inflammation, periportal inflammation/piecemeal necrosis, and lobular inflammation with semiquantitative scores (0 = no activity, 1 = minimal activity, 2 = mild activity, 3 = moderate activity, and 4 = severe activity). The stage of fibrosis was assessed as 0 = no fibrosis, 1 = portal fibrosis, 2 = periportal fibrosis, 3 = septal fibrosis, and 4 = cirrhosis. The main clinical, biochemical, virologic, and histologic features in the patients at the time of liver biopsy are described in Table 1. Healthy liver sections from 3 patients obtained during partial hepatectomy due to metastasis of a nonhepatic primary tumor were used as control.

Immunohistochemical Staining of Tissue Sections and HepG2 Cells. Frozen sections (5 μm) of liver biopsy specimens from patients with different grading of chronic HCV infection were examined. After blocking of endogenous peroxidase with 0.3% hydrogen peroxidase in methanol and washing in phosphate-buffered saline (PBS), nonspecific binding was blocked with 1% BSA for 1 hour. Then, the activation-specific anti–caspase-3 (0.3 μg/mL), anti–caspase-7 (1 μg/mL), and anti-PARP antibodies (0.2 μg/mL) as well as isotype-matched control antibodies were added to the slides in 1% BSA and incubated for 1 hour at room temperature. After repeated washings in PBS, the sections were incubated with the biotinylated secondary antibody (20 μg/mL) for 30 minutes. The sections were washed again and then covered with an avidin-biotin complex reagent containing horseradish peroxidase for 1 hour. Finally, the sections were washed in PBS and stained in a freshly prepared substrate solution (4 mg AEC in 10 mL sodium acetate buffer, pH 4.9, 500 μL dimethylformamide, 0.03% hydrogen peroxide) for 10 minutes. The reaction was stopped by extensive rinsing in dimineralized water. Subsequently, the sections were counterstained with hematoxylin and mounted in glycerol/gelatine. HepG2 cells were stained in a similar way. Briefly, cells were seeded on coated coverslips in 6-well plates (1×10^6 cells/well), treated with anti-CD95 antibody (0.5 μg/mL) and cycloheximide (10 μg/mL), and then fixed in 3.7% paraformaldehyde, before staining was performed with the avidin-biotin complex immunoperoxidase method. The number of immunoreactive cells in the biopsy specimens was assessed by the Openlab image software (Improvision, Coventry, England). Pictures of a 10-fold magnification were imported, and positive cells in 4 microscopic fields of an approximately 400-fold magnification were counted. A statistical analysis comparing the frequency of immunoreactivity was performed using the U-test according to Mann and Whitney. A P value $<.05$ was considered to be significant.

TUNEL Staining. DNA fragmentation was visualized by an enzymatic reaction using the *in situ* cell death detection kit (Roche Molecular Biochemicals). Briefly, sections were permeabilized for 5 minutes on ice with 0.1% Triton-X100 in 0.1% sodium citrate buffer pH 4.7. After a washing step in 50 mmol/L Tris-HCl pH 7.2, the sections were incubated for 1 hour at 37°C in a reaction mixture (200 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl pH 6.6, 0.2 mmol/L ethylenediaminetetraacetic acid and 0.25 mg/mL BSA) containing terminal deoxynucleotidyl transferase (0.2 U/μL) and fluoresceine-labeled deoxyuridine triphosphate (dUTP). After stopping the reaction and washings in PBS, sections were embedded in fluorescence mounting medium (DAKO, Hamburg, Germany). Sections labeled in absence of terminal deoxynucleotidyl transferase were used as negative controls, whereas sections pretreated with DNase I served as positive controls. In double-staining experiments DNA fragmentation was measured by immunofluorescence by using fluoresceine-labeled dUTP in the TUNEL reaction, and anti-cleaved

TABLE 1. Clinical, Virologic, and Histologic Features in Patients

Patient	Sex and Age (yr)	Activation Score	Virus Load (10^6 copies/mL)	HCV Genotype	AST (U/L)	ALT (U/L)
1	M, 40	G0, St1	0.71	NA	26	39
2	M, 38	G0, St1	>1	3a	14	22
3	M, 61	G0, St1	>1	1b	24	42
4	M, 57	G0, St1	>1	NA	21	38
5	F, 40	G1, St0	0.53	1b	15	21
6	M, 35	G1, St1	>1	1b	15	20
7	F, 43	G1, St2	0.83	NA	8	11
8	M, 41	G1, St2	>1	NA	14	28
9	F, 46	G1, St2	0.61	1b	12	15
10	M, 37	G1, St2	0.70	1b	14	25
11	F, 62	G1, St2	0.37	1a	24	39
12	M, 22	G1, St1	>1	2	23	47
13	M, 66	G2, St1	0.07	1b	15	28
14	M, 35	G2, St2	0.29	NA	31	75
15	M, 46	G2, St2	>1	1a	52	95
16	F, 59	G2, St2	NA	1b	25	38
17	F, 56	G2, St2	NA	1b	21	32
18	M, 42	G2, St3	0.02	NA	37	48
19	F, 24	G3, St2	0.003	3a	274	536
20	F, 38	G3, St4	>1	3a	23	27

NOTE. Activation score was determined according to Batts and Ludwig.[22]
Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; G, grade of necroinflammatory activity; St, stage of fibrosis; NA, not available.

caspase-3 and Cy3-conjugated donkey anti-rabbit secondary antibody for the detection of caspase-3 activation.

Flow Cytometry. The relative time course of DNA fragmentation and caspase activation was analyzed by flow cytometric staining of HepG2 cells. Cells were seeded in 6-well plates and treated with anti-CD95 (0.5 μg/mL) in the presence of cycloheximide (10 μg/mL). After the indicated times, cells were harvested and fixed in ice-cold methanol. After 2 washes in PBS, DNA fragmentation was detected by TUNEL staining by using fluoresceine-labeled dUTP as described above. To simultaneously measure caspase activation, cells were incubated with the activation-specific caspase-3 or anti-cleaved PARP antibodies or the respective IgG controls for 1 hour and labeled with phycoerythrin-conjugated secondary antibodies for 30 minutes after the TUNEL reaction. The percentage of positive cells was quantified in a FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany) with CellQuest software using the FL1 channel for TUNEL reactivity and the FL2 channel for caspase activation.

Extracts and Immunoblotting. The proteolytic cleavage of caspases and PARP was investigated by immunoblotting in HepG2 cells. A total of 1×10^6 cells were seeded in 6-well plates and treated with anti-CD95 (0.5 μg/mL) and cycloheximide (10 μg/mL). After 6 hours cells were washed in cold PBS and lysed in 1% Triton X-100, 50 mmol/L Tris-HCl, pH 7.6, and 150 mmol/L NaCl containing 3 μg/mL leupeptin, 3 μg/mL aprotinin, 3 μg/mL pepstatin A and 2 mmol/L phenylmethylsulfonyl fluoride. After centrifugation (10 minutes, 13,000 rpm, 4°C), the cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Amersham, Braunschweig, Germany). Membranes were blocked with 5% milk powder in Tris-buffered saline and then incubated for 1 hour with 1 μg/mL of either anti-cleaved caspase-3, anti-cleaved caspase-7, or antibodies recognizing the full-length forms of the proteins. Membranes were washed 4 times with Tris-buffered saline/0.05% Tween-20 and incubated with the respective peroxidase-conjugated secondary antibodies for 1 hour. After extensive washing, bound antibodies were detected by enhanced chemiluminescent staining.

RESULTS

Detection of Caspase Activation and PARP Cleavage in Apoptotic HepG2 Cells. Human hepatoma HepG2 cells were either left untreated or incubated with the agonistic anti-CD95 antibody to induce apoptosis. Activation of effector caspases and PARP cleavage were investigated by immunocytochemistry by using activation-specific antibodies for caspase-3 and caspase-7 as well as an antibody specific for caspase-cleaved PARP. Whereas normal viable cells showed no immunoreactivity (Fig. 1A-C), HepG2 cells induced to undergo apoptosis clearly stained positively for active caspase-3 (Fig. 1D), caspase-7 (Fig. 1E), and cleaved PARP (Fig. 1F). The immunoreactive cells were mostly apoptotic, as they displayed typical condensation of the cytoplasm and nucleus as well as membrane blebbing. No staining was obtained with isotype-matched control IgG (Fig. 1G and H).

To verify the detection of caspase activation, we prepared cell lysates from HepG2 cells and subjected them to immunoblot analyses with either the different activation-specific antibodies or antibodies detecting also the full-length, unprocessed proteins. In apoptotic cells the antibodies against the active caspases detected cleavage products of 17 and 19 kd corresponding to the large active subunit of caspase-3, and the p20 subunit of caspase-7 (Fig. 2). In contrast no immunoreactivity was detected with extracts of unstimulated cells. Immunoblot analysis with the anti-cleaved PARP antibody gave rise to several unspecific protein bands (data not shown). By using antibodies against the full-length forms of the different proteins both the uncleaved forms and the cleavage products of caspase-3, caspase-7, and PARP were detected (Fig. 2).

To study the time course of caspase activation and its relation to other apoptotic events, we simultaneously measured DNA fragmentation and caspase-3 and PARP cleavage by flow cytometry. After incubation with anti-CD95 for different time points, cells were costained with TUNEL to detect fragmented DNA and with anti-cleaved PARP, respectively. As shown in Fig. 3, PARP cleavage was already strongly visible after 2 hours of apoptosis induction, and 34.9% of the cells stained positively with the antibody. In contrast, TUNEL reactivity was delayed and most strongly detected after 12 hours of anti-CD95 treatment (52.7% positive cells). Similar to anti-

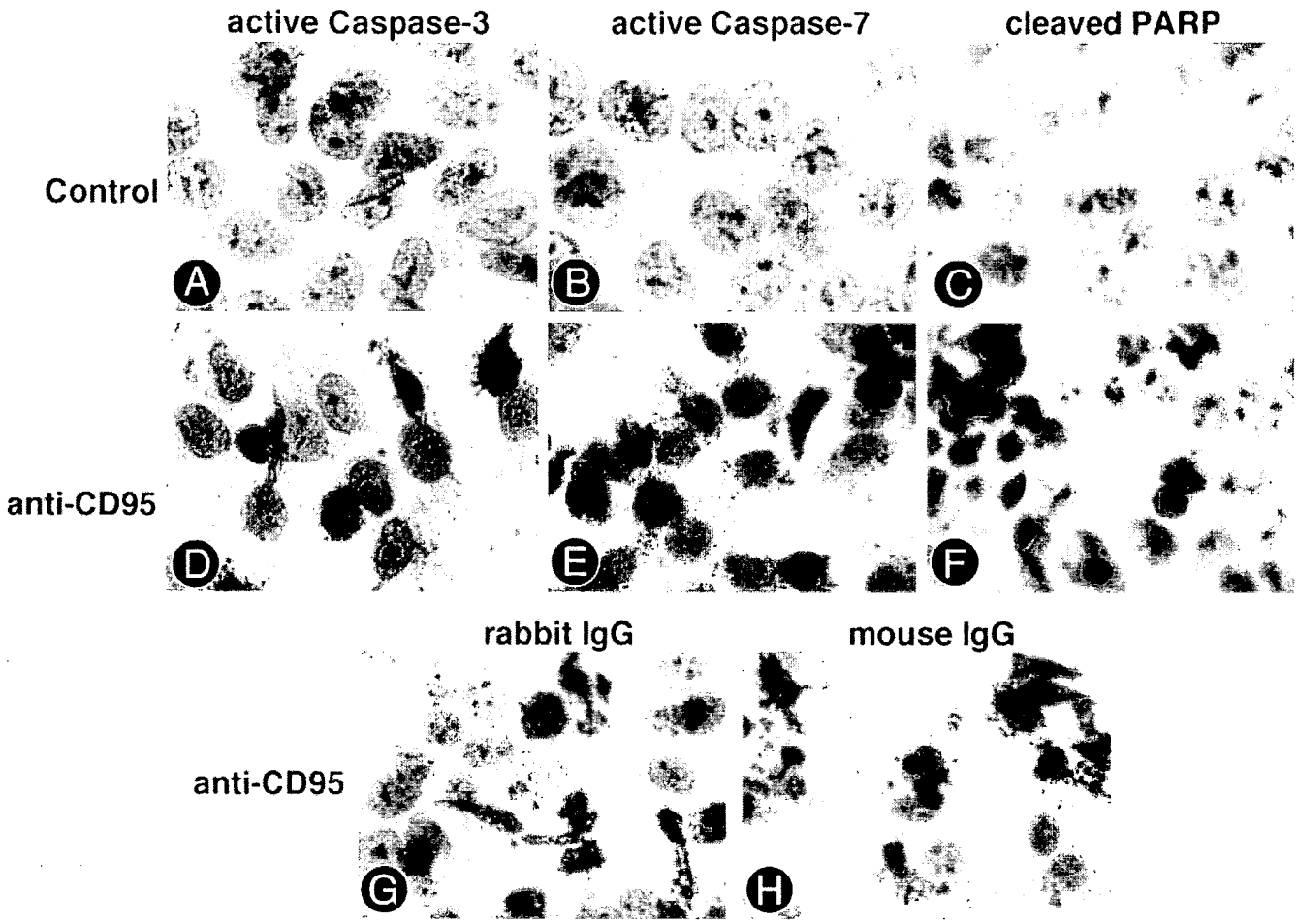

FIG. 1. Detection of caspase activation and PARP cleavage. HepG2 cells were either left untreated (A-C) or incubated with agonistic anti-CD95 for 6 hours to induce apoptosis (D-F). Staining of cells was performed by the immunoperoxidase method by using antibodies specific for active caspase-3 (A and D), active caspase-7 (B and E), and caspase-cleaved PARP (C and F). No staining was observed in viable cells, whereas apoptotic cells revealed a strong immunostaining with the antibodies. Normal rabbit IgG (G) and isotype-matched mouse IgG (H) served as controls.

cleaved PARP, immunoreactivity with anti-active caspase-3 preceded TUNEL staining (data not shown). These results indicate that caspase-3 and PARP cleavage mark early events during apoptosis.

In Situ *Detection of Caspase Activity and PARP Cleavage in Liver Tissue of Patients With Chronic HCV Infection.* Inappropriate apoptosis is presumably involved in HCV-mediated liver injury. This prompted us to investigate the activation of effector caspases and PARP cleavage in liver tissues from patients with chronic HCV infection. Almost no immunoreactivity was evident in healthy liver tissue using antibodies specific for activated caspase-3 and -7 and cleaved PARP (Fig. 4A-C). In contrast, liver tissue from a patient with chronic HCV infection clearly showed hepatocytes that stained positively for active caspase-3 and caspase-7 as well as for cleaved PARP (Fig. 4D-F). Interestingly, active caspase-3 and -7 appeared to have a slightly different subcellular localization. Cleaved PARP and active caspase-3 were predominantly found in the cell nucleus, whereas the anti-active caspase-7 antibody often labeled perinuclear regions. It has previously been observed in mouse liver and Jurkat T-cells that active caspase-7 is associated with microsomal fractions or translocated to the endoplasmic reticulum upon apoptosis induction.[23,24] Moreover, it was noteworthy that many hepatocytes, albeit positive for caspase activation and PARP cleavage, did not exhibit an overt apoptotic nuclear morphology (Fig. 4D-F). Additionally, we performed a double-staining for caspase-3 activation and DNA fragmentation. Interestingly, the majority of cells with caspase-3 activation did not reveal TUNEL reactivity (Fig. 4G-I). Overall, these data further support the idea that immunohistochemical detection of caspase activation and PARP cleavage marks early events in the apoptotic process.

Correlation of Caspase Activation and the Grade of Disease Activity in Liver Biopsy Specimens of Patients With Chronic HCV Infection. We next investigated whether the extent of caspase activation was dependent on the grade of liver inflammation. We therefore studied liver biopsy specimens from patients with different grades of disease activity according to Batts and Ludwig.[22] Patients with disease activity grade 1 (Fig. 5A-C) and grade 2 (Fig. 5D-F) revealed lower levels for caspase-3 (Fig. 5A and D), caspase-7 (Fig. 5B and E), and PARP (Fig. 5C and F) activation, whereas in patients with grade 3 (Fig. 5G, H, and I) a higher expression of activated caspase-3 (Fig. 5G), caspase-7 (Fig. 5H), and PARP (Fig. 5I) was detected. Statistical analysis of 20 patients with different grades of disease activity revealed that the extent of caspase-3 and caspase-7

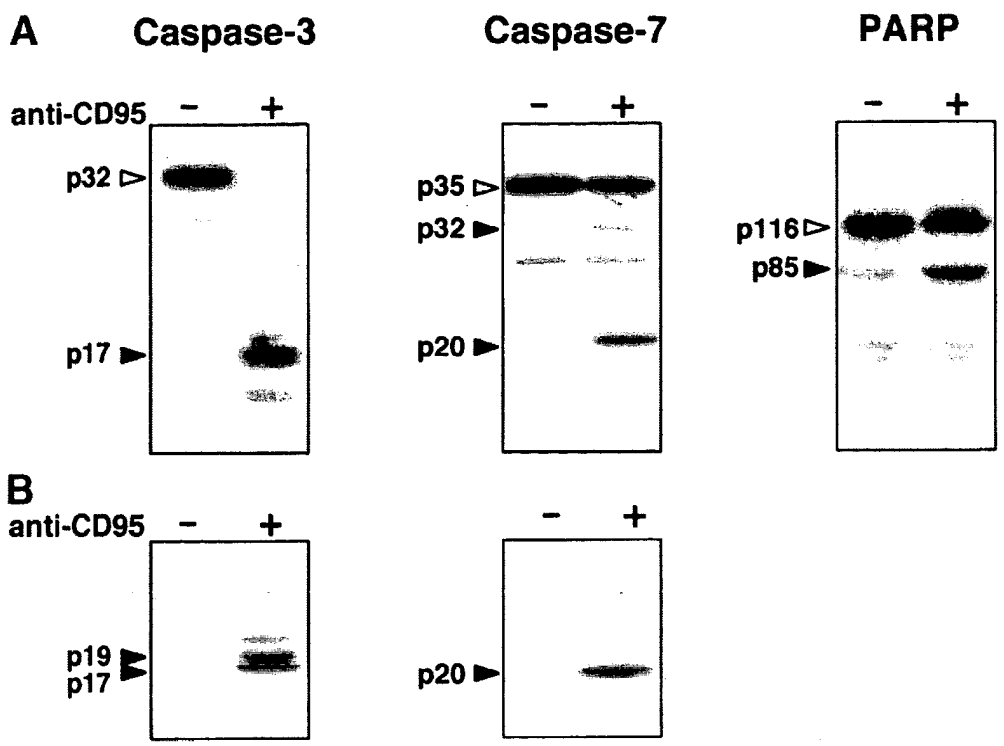

FIG. 2. Immunoblot analysis of caspase activation and PARP cleavage in HepG2 cells. Lysates from HepG2 cells treated for 6 hours in the presence or absence of anti-CD95 were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. (A) The staining pattern of the antibodies directed against the full-length forms of caspase-3, caspase-7, and PARP. Both the unprocessed forms (*open arrowheads*) and the different cleavage products (*closed arrowheads*) were detected. (B) The activation-specific anti–caspase-3 and -7 antibodies selectively detected the large active subunits of caspase-3 and caspase-7, respectively. Immunoblotting with anti-cleaved PARP revealed several unspecific protein bands (data not shown).

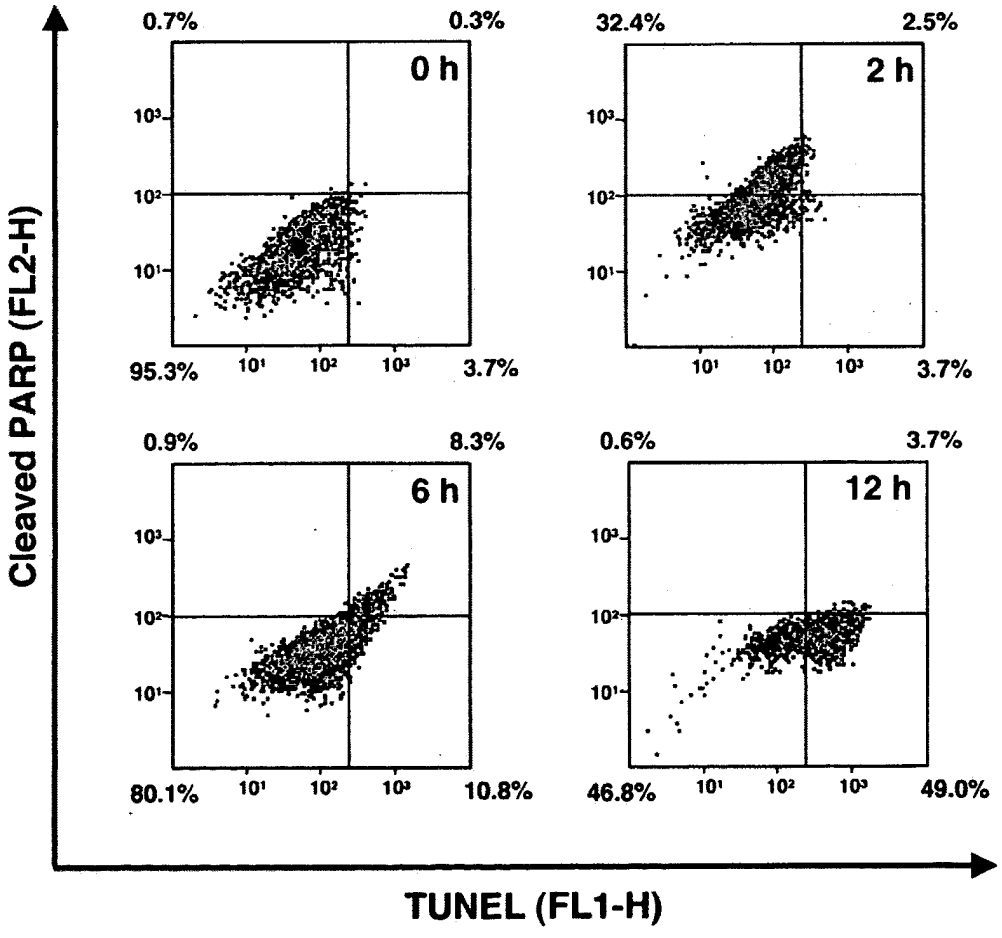

FIG. 3. Relative time course of PARP cleavage and DNA fragmentation. HepG2 cells were incubated for the indicated times with anti-CD95 in the presence of cycloheximide. DNA fragmentation and PARP cleavage were then determined in fixed cells by TUNEL staining and immunostaining with anti-cleaved PARP, respectively. The dot blots of flow cytometric measurements show that PARP cleavage preceded the onset of DNA fragmentation. However, anti-cleaved PARP staining decreased at later stages of apoptosis, whereas TUNEL reactivity was still detectable. No staining was observed in the absence of terminal deoxynucleotide transferase or by using a control IgG instead of anti-cleaved PARP. Like anti-cleaved PARP, also immunoreactivity with anti-cleaved caspase-3 preceded TUNEL staining (data not shown).

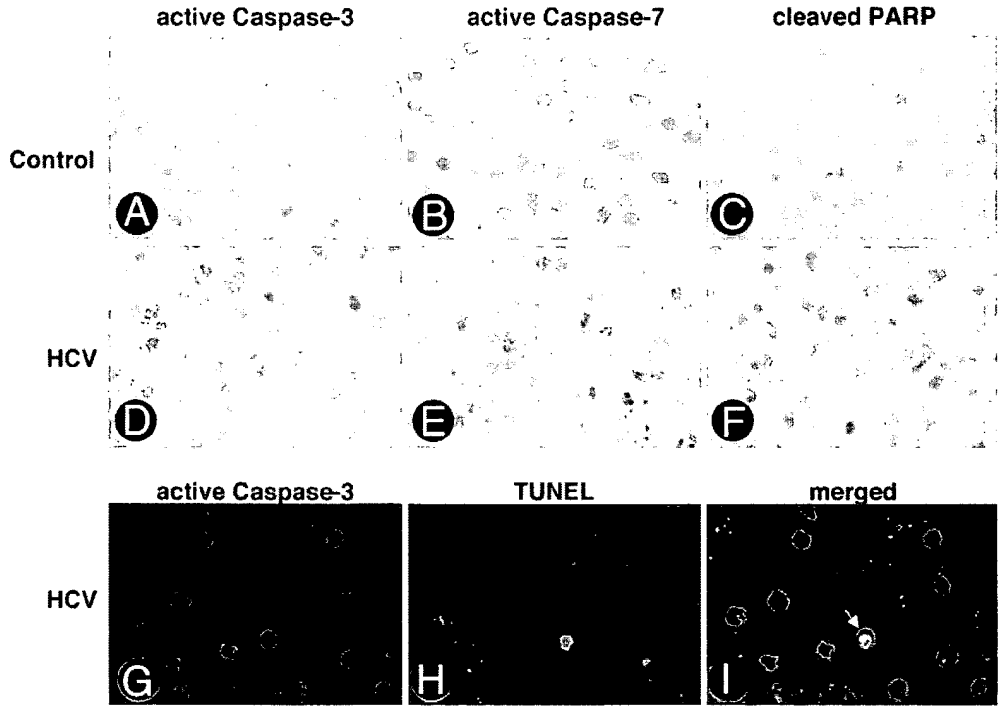

FIG. 4. Immunohistochemical detection of active caspase-3, active caspase-7, and cleaved PARP in liver biopsy specimens. Liver tissues of a control person (A-C) and a grade 2 HCV patient (D-F) were analyzed with the activation-specific antibodies for caspase-3 (A and D), caspase-7 (B and E), and PARP (C and F). Almost no immunostaining was obtained in normal liver, whereas a liver biopsy from the patient with chronic HCV infection showed intense staining with the antibodies (original magnification ×400). Neither was immunostaining detectable with irrelevant control antibodies. Note that the antibodies labeled hepatocytes with apoptotic morphology as well as cells with a rather intact cell nucleus. (G-I) Immunofluorescent double-staining of a liver biopsy specimen from a grade 2 HCV patient with anti-active caspase-3 (G) and TUNEL (H). A composite image (I), in which the green TUNEL staining was superimposed to the red staining of caspase-3 shows that only a small proportion of cells with active caspase-3 revealed DNA fragmentation, as indicated by the merged yellow color (*arrow*).

activation and PARP cleavage correlated positively with the grade of inflammation (Fig. 6A-C). The mean number of cells positive for active caspase-3 was 7.7% ± 2.9% in grade 0, 12.2% ± 3.2% in grade 1, 17.7% ± 3.8% in grade 2, and 20.9% ± 7.4% in grade 3, whereas in control livers not more than 2.1% ± 0.5% of the cells were labeled. Very similar values were obtained when the number of cells reactive for active caspase-7 and cleaved PARP was compared in the different patient groups, underlining the reliability of the correlation to the grade of necroinflammatory injury. In contrast, no staining was obtained by using isotype-matched control antibodies. Regression analysis, in addition, revealed a strict correlation between caspase-3 and caspase-7 activation (Fig. 6D). Unlike the grade of disease, however, no significant correlation between caspase activation and serum transaminase levels (Fig. 7) or the viral load or HCV genotype (data not shown) was found. Thus, the positive correlation of caspase-3 and caspase-7 activation and PARP cleavage with the necroinflammatory activity suggests that apoptosis might be involved in HCV-associated immune response and liver injury.

DISCUSSION

There is increasing evidence suggesting that liver cell damage in chronic HCV infection is mediated by induction of

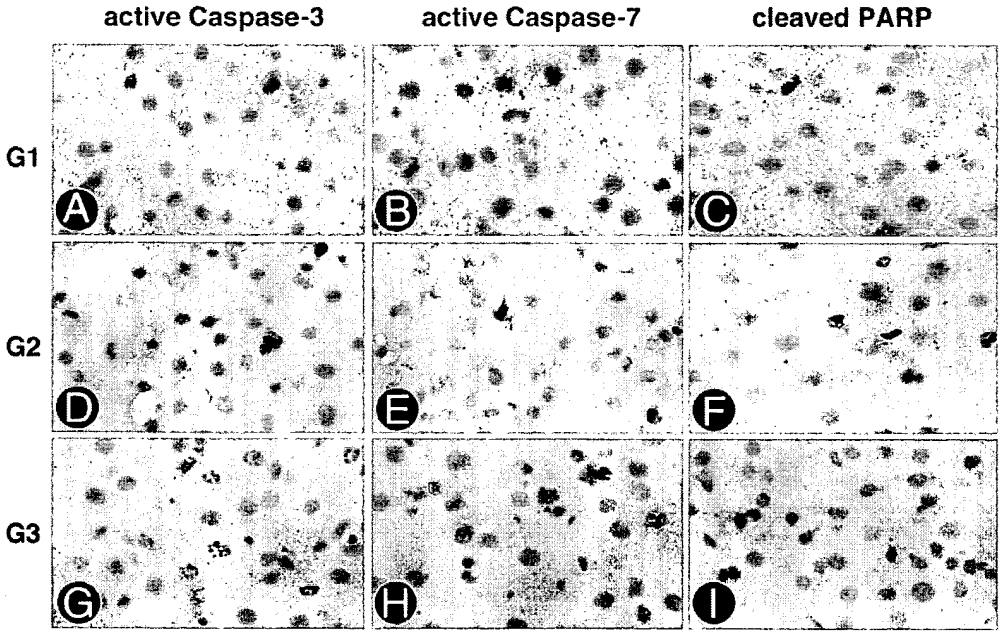

FIG. 5. Detection of caspase activation and PARP cleavage in liver biopsy spcecimens with different necroinflammatory activity (original magnification ×400). Liver biopsy specimens of patients with grade 1 (A-C), grade 2 (D-F), and grade 3 (G-I) disease activity according to Batts and Ludwig[22] were immunostained with antibodies specific for active caspase-3 (A, D, and G), caspase-7 (B, E, and H), and cleaved PARP (C, F, and I). Note that immunoreactivity is elevated in liver biopsy specimens from patients with higher necroinflammatory activity.

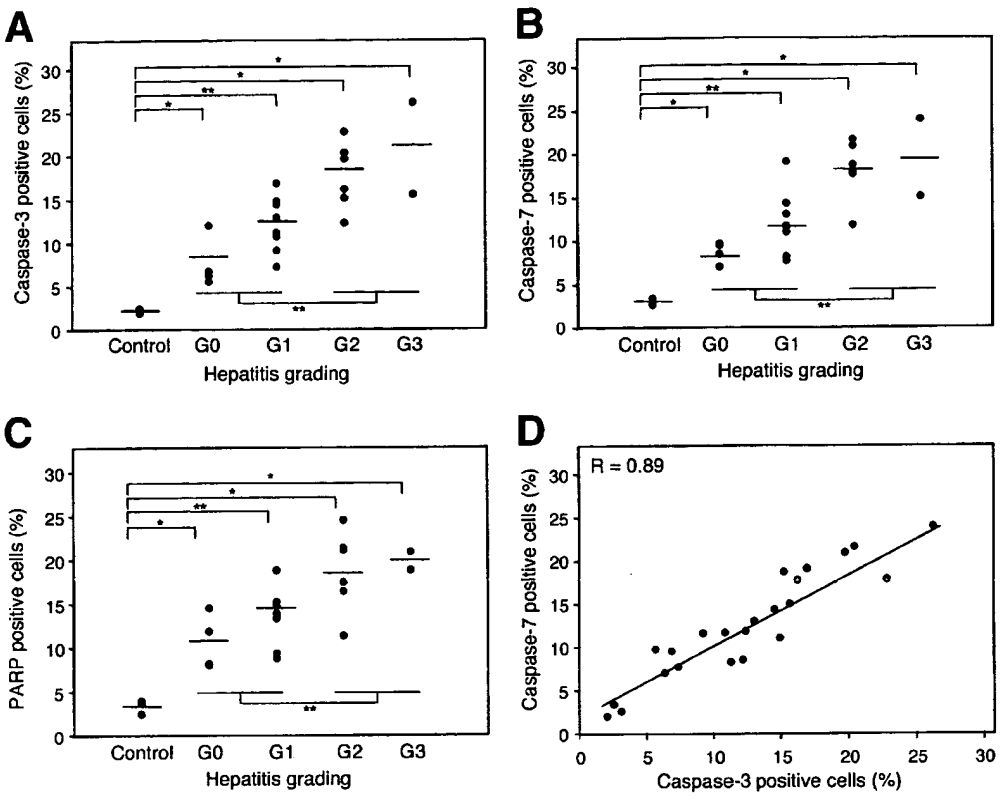

FIG. 6. Correlation of caspase activation and PARP cleavage activity with the grade of liver disease activity. Biopsy specimens from 20 patients with chronic HCV infection and different grades of necroinflammatory activity and from 3 control individuals were stained with the activation-specific antibodies for caspase-3 (A), capase-7 (B), and PARP (C). The number of positive cells was assessed by counting 4 microscopic fields at a 400-fold magnification. (D) A regression analysis correlating caspase-3 with caspase-7 activity. *Horizontal bars* indicate the mean number of positive cells within each group. $*P < .05$; $**P < .01$.

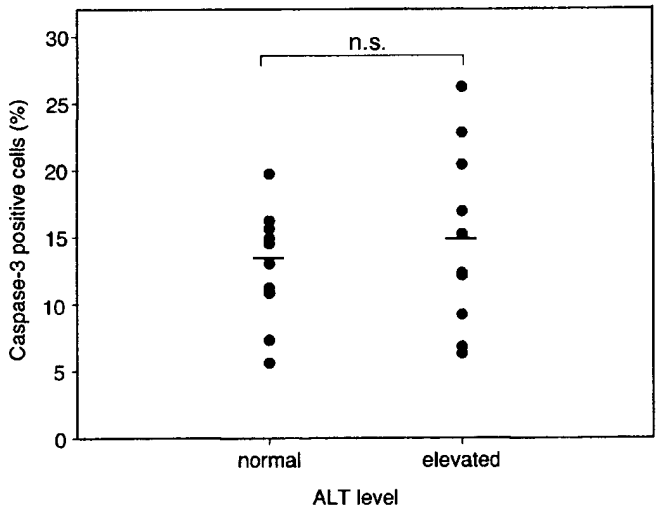

FIG. 7. Caspase-3 activation in biopsy specimens of HCV-infected patients with normal (n = 10) and elevated (n = 10) serum aminotransferase activity. Liver biopsy specimens were stained with the activation-specific antibody against caspase-3, and the number of positive cells was calculated as described in Fig. 5. Alanine transaminase values were considered as elevated, if the patients had increased serum levels (men >29 U/L, women >19 U/L) on at least 3 different occasions during the last 6 months. *Horizontal bars* indicate the mean number of positive cells showing no substantial difference between both groups. Neither was a significant correlation observed when the number of cells positive for active caspase-7 or cleaved PARP was compared with the aminotransferase levels (data not shown).

apoptosis. However, the relative contribution of either apoptosis or necrosis as well as the functional role of caspases and their involvement in liver damage are largely unknown. The importance of apoptosis in HCV infection has originally been proposed in view of pathomorphologic features including (1) cell shrinkage and fragmentation of the nucleus, in particular in areas of piecemeal necrosis, (2) the presence of acidophilic bodies, and (3) focal cell dropouts in the liver lobule, which are features of apoptosis of individual infected hepatocytes.[25] However, the number of cells with a clear apoptotic morphology in the liver of patients with HCV infection is relatively small, and also biochemical techniques, such as TUNEL staining, which detects apoptotic DNA fragmentation, failed so far to show apoptotic events in a considerable proportion of hepatocytes in HCV infection.

In the present study, we investigated the activation of caspases, which are the central executioners of many, if not all apoptotic pathways. Caspase-3 and caspase-7 are two main executioner caspases that cleave several substrates including PARP and finally lead to apoptotic cell death. Mainly by means of immunoblot analysis both caspases were shown to be activated in mouse hepatitis models including tumor necrosis factor α– and CD95L-induced liver disease.[23,26] The importance of caspases is underscored by studies with pharmacologic caspase inhibitors, which potently suppressed experimental hepatitis, as well as by the phenotype of caspase-3 knockout mice that are largely resistant against CD95-induced liver damage.[27-29]

Three points of our present study deserve particular attention: Our results show that caspases are indeed activated in human biopsy specimens of chronic hepatitis patients. Second, we show that the activation of caspases correlates signif-

icantly with the inflammatory activity but not with other surrogate markers of HCV infection, such as serum transaminase levels or viral load. Third, unlike studies investigating late apoptotic features, we found that the activation of caspases is detectable in a considerable percentage of hepatocytes.

Previously, the occurrence of apoptosis in chronic hepatitis has mainly been studied by morphologic criteria as well as the widely used TUNEL technique. In these studies, the amount of liver cell apoptosis as assessed by DNA fragmentation was elevated compared with healthy liver, but was rather low and never exceeded 0.5% of apoptotic hepatocytes in viral hepatitis.[30,31] In contrast, by using 3 independent markers, *i.e.*, caspase-3 and caspase-7 activation and PARP cleavage, we found that caspase activation was considerably higher and, depending on the grade of disease activity, detectable in approximately 7% to 20% of liver cells. There are several possibilities that may account for these differences to previous studies. In tissue biopsy specimens, it is well known that the TUNEL technique has severe limitations and may not permit a reliable quantification of apoptotic cells. Several concerns have been expressed about pitfalls, lack of specificity, and difficulties to standardize the TUNEL technique.[32-35] Furthermore, it is a common experience that performance of TUNEL staining depends greatly on the tissue pretreatment and labeling procedures.[36] Another reason for the discrepancy in the number of TUNEL-positive cells and cells revealing caspase activation may be explained by the different time course of biochemical events in apoptosis. DNA fragmentation is recognized as a late event in apoptosis, whereas caspase activation occurs earlier than DNA cleavage. It has been also found that some forms of apoptotic cell death, even in hepatocytes, are not always associated with DNA fragmentation.[37,38] Finally, it is conceivable that in late stages of apoptosis, when DNA fragmentation occurs, apoptotic cells are rapidly phagocytosed by neighboring macrophages and therefore escape detection by TUNEL staining. Thus, the approach of quantifying apoptosis by morphologic criteria and DNA fragmentation may underestimate the number of cells undergoing apoptosis.

It has been reported that liver injury in chronic HCV infection is not directly related to either the number of infected hepatocytes or the serum HCV RNA concentration.[39] Kinetic analysis of viral turnover in patients indicated that HCV infection is a highly dynamic process with a short half-life of viral particles and HCV-infected cells.[40] It has been calculated that the daily turnover of HCV-infected cells may be as high as 13% to 25%. Assuming that in patients with chronic HCV infection approximately 50% of hepatocytes are infected, this would mean that between 6.5% and 12.5% of hepatocytes are killed daily.[31,41] It is remarkable that this number is very similar to the amount of cells with active caspases ranging from 7% to 20%.

It is commonly assumed that activation of the caspase cascade marks a lethal hit and "point-of-no-return" in the apoptotic pathway. However, the identification of endogenous caspase inhibitors and some other recent evidence suggest that within a cell the extent of caspase activation may be restricted and not necessarily lead to cell death. It has been shown that caspases, including caspase-3, are transiently activated upon cell stimulation in nonapoptotic, proliferating T cells.[42,43] Importantly, in these cells caspase activity resulted in a rather selective substrate cleavage, because PARP and lamin B, but not DNA fragmentation factor or replication factor C were processed. It was also shown that caspase activation is required for erythrocyte differentiation without inducing cell death.[44] Thus, it is possible that the hepatocytes immunoreactive for active caspase-3 and -7 either undergo apoptosis or, despite the activation of caspases, do survive.

Similar to previous studies investigating DNA fragmentation in HCV infection,[30,31] we did not find a significant correlation of caspase activation to viremia or serum transaminase levels. This lack of correlation may be related to different types of cell death. *In vivo* studies have shown that apoptosis of hepatocytes was accompanied by elevated transaminase levels, but that the release of transaminases was lower in apoptosis than in necrosis.[1] Thus, relative differences in the occurrence of apoptosis and necrosis could explain why transaminase levels and caspase activation are not correlated. Our results further suggest that chronic liver damage and hepatocyte cell loss by apoptosis can occur in HCV-infected patients without overt biochemical changes. This may explain the progressive nature of HCV infection that can be seen in asymptomatic patients with normal transaminase levels.

In contrast to these surrogate markers, we found a significant correlation of caspase activation and the histologic grade of disease, *i.e.*, necroinflammatory activity. Patients with grade G0 revealed caspase-3 and caspase-7 activation and PARP cleavage in 7.7%, 8.7%, and 10.7% of the hepatocytes, respectively. In contrast, in G3 hepatitis 20.9%, 19.5%, and 19.9% of cells were found to stain positively with the activation-specific antibodies. In healthy liver tissues all 3 markers never labeled more than 2.6% of the cells. In addition to inflammatory activity, the classification according to Batts and Ludwig[22] provides a semiquantitative measure for the degree of fibrosis. Although apoptosis may be implicated in fibrosis and liver regeneration, we could detect a positive correlation between caspase activation and fibrotic reactions only in lower (0-2) but not in higher stages (3-4) of fibrosis (data not shown). One reason for this observation could be that with increasing fibrotic alterations and cirrhosis the antiapoptotic molecule Bcl-2 is up-regulated which might also explain the high incidence of hepatocellular carcinoma in patients with cirrhosis.[45]

Both immune-mediated reactions and direct cytopathic effects of HCV have been implicated in liver damage. Recent studies showed that the HCV core protein exerts both proapoptotic and antiapoptotic properties in different experimental conditions.[9-12] The HCV core protein can modulate the immune response by activation of transcription factors, such as nuclear factor-κB and activator protein-1, thereby inducing the production of proinflammatory cytokines.[46] Thus, HCV proteins might modulate hepatocyte apoptosis by indirect rather than by direct mechanisms. In line, it was shown that the HCV-specific cytotoxic T-lymphocyte response was associated with lower levels of viremia and higher disease activity.[47] An important mediator of the cytotoxic T-lymphocyte response is CD95L transducing apoptotic signals to hepatocytes. Up-regulation of CD95 as well as induction of CD95L expression on T lymphocytes has been found to correlate with more severe inflammation in HCV infection.[2,7,8,48,49] It was shown that CD95L may also exert proinflammatory activities by inducing interleukin 1β secretion that is responsible for

neutrophil infiltration.[50] Thus, the HCV-mediated immune response is closely associated with CD95-triggered hepatocyte apoptosis, giving rise to several amplification loops.

In summary, our data clearly show that caspase activation is associated with inflammatory reactions, supporting the hypothesis of an immune-mediated mechanism for apoptotic liver cell damage in chronic HCV infection. The positive correlation between caspase activation and inflammatory liver damage in HCV infection opens challenging possibilities for the development and use of new therapeutic agents. A number of small-molecule caspase inhibitors with demonstrated liver efficacy and bioavailability have been identified and are currently being tested in animal models. Monitoring caspase activation might therefore provide a reliable diagnostic tool to detect the degree of HCV-mediated inflammatory liver damage and to evaluate the efficacy of HCV therapy.

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